TRANSLATIONAL PROTEIN-MODIFICATIONS IN DIABETES MELLITUS

PHD THESIS

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Pécs, 2023

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1. Introduction

The estimated number of people suffering in diabetes mellitus in 2013 was 382 million, which presumably will grow even to 592 million by the year of 2035 (1.) The fact that the number of type 2 diabetes mellitus (T2DM) is increasing among children (2) leads to the alteration of the 1:20, T1DM:T2DM ratio, known so far. T2DM leads to a variable metabolic disturbances resulted partially from decreased insulin-secretion, partially from impaired response to insulin, or -in some cases- from the result of both (3). One of the well-known factors in the background of the increased mortality in diabetes mellitus, is insulin resistance, where the impaired effect of insulin results in the abnormally increased level of the hormon. In most of the cases the impaired insulin-response in the liver, adipose tissue and muscle i.e. the peripheral insulin resistance, develops long before hyperglycaemia (4). Insulin resistance in muscle leads to decreased glucose uptake, increased glucose-output from the liver and to the increased output from adipose tissue as well. The chronically increased level of free-fatty acids leads also to the destruction of pancreatic beta cells (lipotoxicity).

Paratyrosine is produced enigmatically and Phe is used by the enzyme as a substrate. Production of o- or m-Tyr is a non-enzymatic reaction in oxidative stress due to hydroxyl free radical.

In our work, we examined the incorporation of the above-mentioned amino-acids into the cellular proteins, their effect on glucose-uptake and insulin-signalling, namely on the phosphorylation of insulin-receptor substrate-1 (IRS-1) and Akt.

2. Aims

Proving the role of the modified phenylalanine derivatives in insulin-resistance.

3. Materials and methods

3T3-L1 mouse fibroblasts, HEK-293 immortalised epithelial cells and BALB/C monocyte-macrophage cells were grown on media containing p-, o-, m-Tyr, 5 or 25 mmol/l glucose. We examined the glucose-uptake of adipocytes by using isotope-labelled glucose, followed by the examination of time- and concentration dependence of glucose uptake. We examined the incorporation of different tyrosines into all the three cell-lines, in both the protein-bound and non-protein bound fraction of cells lysates, with HPLC method, thereafter we examined the effect of p-, o- and m-Tyr, 5 and 25 mmol/l glucose concentration on the phosphorylation of Akt and IRS-1, with Western-blot analysis. For statistical analysis we used SPSS Statistics 27 and GraphPad Prism vs 8 (IBM, IL, USA). The experiments were performed at n=5-10 for each individual measurements. Normality was determined with Kolmogorov-Smirnoff test, followed by different post-hoc analysis.

4. Results

4.1.Ortho- and Meta-Tyr Inhibit Insulin-Induced Glucose Uptake

The insulin induced deoxy-D-glucose 2-[1 2-3H(N)] uptake of 3T3-L1 adipocytes, grown on 5mmol/l glucose and p-Tyr containing medium, was significantly higher at

all used insulin concentrations (2, 20, 200, 400) compared to the control (untreated p-Tyr, 5mmol/l), and was growing insulin concentration-dependently. Cells, grown on 25 mmol/l, p-Tyr containing medium or on media containing equimolar amount of m- or o-Tyr, became insulin resistant, without significant glucose uptake at any used insulin concentrations, compared to the identical controls.

4.2.The inhibitory effect of o-, and m-Tyr on deoxy-D-glucose 2-[1 2-3H(N)] uptake, is time-dependent

3T3-L1 adipocytes were grown on media containing m-, or o-Tyr for 1, 2, 3, 4, 5 or 12 days. Even after one day of culturing, glucose uptake was impaired significantly compared to cells, grown on 5 mmol/l glucose containing medium.

4.3.The inhibitory effect of o-, and m-Tyr on deoxy-D-glucose 2-[1 2-3H(N)] uptake, is concentration dependent

Glucose uptake was measured in cells, grown on media, containing equimolar amount of p- and o-; or p- and m-Tyr. Our results showed that o- and m-Tyr led to significantly decreased glucose uptake compared to p-Tyr controls (p 0.05 vs p-Tyr), even in insulin-untreated cells and lacked further significant increase on insulin treatment, while adding increasing concentration of p-Tyr to the media prevented this effect.

4.4.o- and m-Tyr were transported by adipocytes within several minutes and were built into cellular proteins

o- and m-Tyr may alter insulin signalling by being incorporated into proteins, after cellular uptake of these amino acids. Therefore, we tested whether these amino acids could be transported into the fat cells. Thus, non-protein-bound intracellular p-Tyr content, also the m-Tyr/p-Tyr and o-Tyr/p-Tyr ratios were measured. We detected a continuous uptake of amino acids independently of glucose concentration (5 or 25 mmol/L) and the presence of insulin.

4.5.Both o- and m-Tyr could be incorporated in the intracellular proteins of 3T3-L1 cells

After culturing on p-, o-, or m-Tyr containing media for 12 days, we examined the protein-bound tyrosine content of adipocytes. The pTyr/Phe ratio decreased in cells, grown on m-, or o-Tyr, while the protein-bound o-Tyr/p-Tyr and m-Tyr/p-Tyr ratio significantly increased according to the tyrosine content of the medium.

4.6.Both o- and m-Tyr could be incorporated in the intracellular proteins of HEK-cells

The protein-bound p-Tyr/Phe content of HEK cells, grown on o- or m-Tyr containing media didn't differ significantly from those, grown on p-Tyr, while the o-Tyr/p-Tyr ratio was significantly higher in cells grown on o-Tyr, whereas the m-Tyr/p-Tyr ratio was the highest in cells grown on m-Tyr.

4.7. Both o- and m-Tyr could be incorporated in the intracellular proteins of macrophages

Our experiments on macrophages showed, that the p-Tyr/Phe content of the lysates didn't differ in cells, grown on p-, o- or m-Tyr containing media. The m-Tyr/p-Tyr ratio was the highest in cells grown on m-Tyr. Interestingly enough, the o-Tyr/p-Tyr ratio was the highest in cells grown on m-Tyr, not on o-Tyr containing medium.

4.8. Culturing 3T3-L1 adipocytes in o-, m-Tyr or 25 mmol/l glucose containing medium leads to changes in the activating phosphorylation (Tyr612) of insulin substrate-1 (IRS-1)

In 3T3-L1 adipocytes, cultured in 5 mmol/l glucose containing medium, the insulin-induced phosphorylation of IRS-1 is more then 300% percent of the basal phosphorylation, which is statistically significant ($p<0.05$). In cells, grown on o- or m-Tyr, the phosphorylation of IRS-1 didn't grow significantly even after treatment with 400 nmol/l insulin.

4.9. The phosphorylation of Akt (Ser 473) on insulin treatment in cells, grown on 5- or 25 mmol/l glucose or p-, o-, or m-Tyr

In normal, 5 mmol/l glucose containing medium the phosphorylation of Akt grows significantly, almost fourfold. Neither on 25 mmol/l glucose nor on m- or o-Tyr containing medium could the insulin treatment trigger significant increase in the phosphorylation of Akt. The same time, the basal phosphorylation of Akt without insulin treatment, was significantly higher in cells grown on 25 mmol/l glucose, m-, or o-Tyr containing medium, compared to the cells, grown on 5 mmol/l glucose, p-Tyr containing medium.

4.10. The activating (Tyr612) phosphorylation of IRS-1 changes in o-, m-Tyr or 25 mmol/l glucose containing medium in HEK-cells

In HEK cells, grown on p-Tyr, 5 mmol/l glucose containing medium, the insulin induced activating phosphorylation (Tyr612) of IRS-1 increased to 200% compared to the basal phosphorylation which seemed to be significant ($p<0.05$). In cells, grown on m- or o-Tyr the activating phosphorylation (Tyr612) of IRS-1 doesn't grow significantly after 400 nmol/l insulin treatment, which shows the lack of insulin effect.

4.11. The phosphorylation of Akt (Ser473) on insulin treatment in HEK-cells, grown on p-, m-, or o-Tyr containing media in normal and high glucose containing media

In physiological, 5 mmol/l glucose containing medium, the phosphorylation of Akt (Ser-473) grew significantly after 400 nmol/l insulin treatment ($p<0.05$). Neither on 25 mmol/l glucose, nor on m-, or o-Tyr, could the insulin treatment exert proper effect, i.e. no significant increase in the Akt phosphorylation could

have been detected. In cells grown on 25 mmol/l glucose, m-, or o-Tyr containing medium, the basal phosphorylation was significantly higher than in control cells.

4.12. The activating (Tyr612) phosphorylation of IRS-1 changes in o-, m-Tyr or 25 mmol/l glucose containing medium in macrophages

In macrophages grown on p-Tyr, 5 mmol/l glucose containing medium, insulin induced activating phosphorylation (Tyr612) of IRS-1 increased to more than 200% compared to the basal phosphorylation which seemed to be significant ($p<0.05$). In cells, grown on m- or o-Tyr or on 25 mmol/l glucose the activating phosphorylation (Tyr612) of IRS-1 didn't grow significantly after 400 nmol/l insulin treatment, which shows the lack of insulin effect. In cells grown on 25 mmol/l glucose, m-, or o-Tyr containing medium, the basal phosphorylation was significantly higher than in control cells.

4.13. The phosphorylation of Akt (Ser473) on insulin treatment in macrophages, grown on p-, m-, or o-Tyr containing media in normal and high glucose containing media

In physiological, 5 mmol/l glucose containing medium, the phosphorylation of Akt (Ser-473) grew significantly after 400 nmol/l insulin treatment ($p<0.05$). Neither on 25 mmol/l glucose, nor on m-, or o-Tyr, could the insulin treatment exert proper effect, i.e. no significant increase in the Akt phosphorylation could have been detected. Basal phosphorylation in 25 mmol/l glucose, m-, or o-Tyr containing media was significantly higher than in control cells.

5. Discussion

We showed in our experiments that abnormal amino-acids: o- and m-Tyr, which are produced in oxidative stress, can inhibit the glucose uptake of adipocytes. We also showed that the signal transduction of cells grown in o- or m-Tyr can change. This phenomenon has been shown on three different cell lines. We also showed in the cases of these three cell lines that o- and m-Tyr could be transported by the cells and incorporated in cellular proteins.

The connection between insulin resistance end oxidative stress has widely been studied. According to our opinion the pathophysiological changes that can be detected in insulin resistance are not only due to the background oxidative stress but also due to the incorporation of pathological amino acids into proteins, which finally leads to changes of signal transduction processes.

Phe, an essential amino acid, takes part in the formation of several clinically significant amino acids and biomarkers, is one of the most reactive target molecule in oxidative stress [5]. It is well known that in the presence of superoxid, H_2O_2 is formed into hydroxyl free radical [6]. By the contribution of hydroxyl free radicals and ionization, Phe residues of proteins can be turned to aphysiological o- and m-Tyr in non -enzymatical processes [7]. Thus, o- and m-Tyr can be considered as markers of oxidative stress [8] and on the basis of their fluorescence, after proper sample preparation their amount could be detected, with fluorescence detector [9].

While our experiments lacked any kind of obvious oxidative agent, araises the possibility that o- and m-Tyr are not only the markers of hydroxyl induced oxidative stress but also molecules with own biological activity. In our experiments we found that o- and m-Tyr could be taken up by adipocytes and could be incorporated into cellular proteins.

In our experiments we detected decreased glucose uptake in adipocytes from the first day both on o- or m-Tyr containing media. These observations refer to a rapid and direct effect of o- and m-Tyr. The glucose uptake decreased significantly even after one day of culturing on o- or m-Tyr containing media and lacked further changes with longer culturing time. This shows that the pathological Tyr isomers might be incorporated to the place of physiological Phe, even after one day and thus can change signal transduction. Parallel to these results in our experiments we found that in adipocytes grown on media containing o- or m-Tyr the insulin induced glucose uptake is impaired, similarly in cells grown in high glucose containing media, i.e. the cells become insulin resistant. Based on our experiments we suppose that the impaired glucose uptake of adipocytes is due to the direct effect of o- and m-Tyr.

In our experiment we observed in three different cell lines that the o- and m-Tyr supplementation of the media leads to insulin resistance likewise in high glucose containing circumstances. The IRS-1 phosphorylation of cells, grown on o- or m-Tyr containing media, increased even without insulin treatment, but this couldn't grow further in the presence of insulin which refers to insulin resistance. The phosphorylation of Akt changed similarly.

Summing up, in our work we showed an association between the incorporation of o- and m-Tyr into cellular proteins and the signal transduction of insulin. Unfortunately, there is no commercially available antibody that attaches specifically to o- or m-Tyr, or even to proteins, consisting these amino-acids, thus their presence in crucial signal-transduction proteins cannot be shown directly.

Despite all these limitations, our experiments strongly suggest that o- and m-Tyr can be incorporated into cellular proteins of adipocytes. The incorporation leads to disturbance in insulin signal-transduction similarly that can be detected in high glucose circumstances.

6. Conclusions

In our experiments on 3T3-L1 adipocytes, we found that in cells, grown on oxidised amino-acids: namely o- and m-Tyr containing media, insulin-induced glucose uptake is impaired significantly, i.e. the cells become insulin resistant. In the background of this phenomenon, we supposed and eventually proved, that o- and m-Tyr is transported in the cells and incorporated into cellular proteins. It has also been shown that these transport processes are independent of insulin. The incorporation of these amino acids into crucial proteins of cellular signal transduction, could be a possible explanation of insulin resistance. This has also been confirmed by our results, in three cell lines, that an impaired protein phosphorylation of IRS-1 and Akt take part in insulin signalling disturbances. We found that insulin resistance develops already after one day of culturing. These effects could have been prevented by adding three-fold p-Tyr in case of o-Tyr and fourfold p-Tyr in case of m-Tyr to the culturing media, which refers to the fact

that these amino acids might be in competition with p-Tyr. If so, that can be a future target process in the pharmacological treatment of insulin resistance. Based on these observations, our research group has an accepted patent [10].

7. Main findings of the thesis

- o- and m-Tyr are taken up by the cells
- o- and m-Tyr are incorporated into cellular proteins
- Insulin-induced glucose uptake is impaired in 3T3-L1 adipocytes due to the effect of o-and m-Tyr
- The effect of o- and m-Tyr on glucose-uptake can be prevented by p-Tyr
- In 3T3-L1 adipocytes, in HEK-cells and in macrophages, the insulin-free, basal phosphorylation of IRS-1 is higher, while insulin-induced phosphorylation of IRS-1 is lacking due to 25 mmol/l glucose o- or m-Tyr, i.e., the cells become insulin resistant
- In 3T3-L1 adipocytes, in HEK-cells and in macrophages, the insulin-induced phosphorylation of Akt is impaired due to 25 mmol/l glucose, o,- or m-Tyr

8. Publications

8.1. Article related to the thesis.

Judit Mohás-Cseh, Gergő Attila Molnár, Marianna Pap, Boglárka Laczy, Tibor Vas, Melinda Kertész, Krisztina Németh, Csaba Hetényi, Orsolya Csikós, Gábor K. Tóth, Attila Reményi and István Wittmann. Incorporation of Oxidized Phenylalanine Derivatives into Insulin Signaling Relevant Proteins May Link Oxidative Stress to Signaling Conditions Underlying Chronic Insulin Resistance

BIOMEDICINES 2022 Apr 22;10(5):975. IF: 4.757.

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8.2. Article, unrelated to the thesis

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